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ORIGINAL RESEARCH ARTICLE

Towards integrated control of varroa: effect of variation in hygienic behaviour among honey bee colonies on mite population increase and deformed wing virus incidence

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Summary

Hygienic behaviour in the honey bee, *Apis mellifera*, is the uncapping and removal of dead, diseased or infected brood from sealed cells by worker bees. We determined the effect of hygienic behaviour on varroa population growth and incidence of deformed wing virus (DWV), which can be transmitted by varroa. We treated 42 broodless honey bee colonies with oxalic acid in early January 2013 to reduce varroa populations to low levels, which we quantified by extracting mites from a sample of worker bees. We quantified varroa levels, again when the colonies were broodless, 48 weeks later. During the summer the hygienic behaviour in each colony was quantified four times using the Freeze Killed Brood (FKB) removal assay, and ranged from 27.5 % to 100 %. Varroa population increased greatly over the season, and there was a significant negative correlation between varroa increase and FKB removal. This was entirely due to fully hygienic colonies with >95 % FKB having only 43 % of the varroa build up of the less hygienic colonies. None of the 14 colonies with >80 % FKB removal had overt symptoms of DWV, whilst 36 % of the less hygienic colonies did. Higher levels of FKB removal also correlated significantly with lower numbers of DWV RNA copies in worker bees, but not in varroa mites. On average, fully hygienic colonies had c. 10,000 times less viral RNA than less hygienic colonies.

Hacia el control integrado de varroa: efecto de la variación en el comportamiento higiénico entre colonias de abejas de la miel con aumento de la población de ácaros y de la incidencia de virus de las alas deformadas

Resumen

El comportamiento higiénico en la abeja de la miel, *Apis mellifera*, se basa en el desoperculado y la eliminación de la cría muerta, enferma o infectada a de las celdas selladas por las abejas obreras. Se determinó el efecto del comportamiento higiénico en el crecimiento de la población de varroa y la incidencia del virus de las alas deformadas (VAD), que puede ser transmitido por la varroa. Se han tratado 42 colonias de abejas de la miel sin larvas con ácido oxálico a principios de enero de 2013 para reducir las poblaciones de varroa a niveles bajos, lo que fue cuantificado mediante la extracción de los ácaros de una muestra de las abejas obreras. Se cuantificaron los niveles de varroa, de nuevo cuando las colonias no tenían cría, 48 semanas después. Durante el verano, el comportamiento higiénico en cada colonia se cuantificó cuatro veces utilizando el ensayo de congelar la cría para matarla (BCM), y este varió entre el 27,5% y el 100%. La población de Varroa aumentó considerablemente durante la temporada, y se observó una correlación negativa significativa entre el aumento de la varroasis y la

eliminación por BCM. Esto se debió enteramente a colonias totalmente higiénicas con > 95% BCM que tuvieron menos varroa que las colonias menos higiénicas. Ninguna de las 14 colonias con > 80% de eliminación de BCM tuvieron síntomas manifiestos de VAD, mientras que el 36% de las colonias menos higiénicas sí que lo tuvieron. Los niveles más altos de eliminación BCM también se correlacionaron significativamente con un menor número de copias de ARN del VAD en las abejas obreras, pero no en los ácaros varroa.

Keywords: *Varroa destructor*, hygienic behaviour, social immunity, honey bee, *Apis mellifera*, DWV, deformed wing virus

Introduction

Honey bees, *Apis mellifera*, face many threats (Ratnieks and Carreck, 2010, Carreck *et al.*, 2010; Harz *et al.*, 2010; Potts *et al.*, 2010). Probably the most serious is the parasitic mite *Varroa destructor*, which can harm colonies both directly, by damaging individual worker pupae so that the resulting adult's lifespan and body weight are reduced (van Dooremalen *et al.*, 2012), and indirectly by transmitting virus diseases (Ball and Allen, 1988; Guzmán-Novoa *et al.*, 2010; Boecking and Genersch, 2008; Highfield *et al.*, 2009). There has been considerable research on varroa control, including synthetic chemicals (Alonso de Vega *et al.*, 1990), natural chemicals such as oxalic acid (Nanetti *et al.*, 2003), biotechnical methods such as drone brood trapping (Charriere *et al.*, 2003, Calderone, 2005), and natural resistance such as hygienic behaviour (Spivak, 1996; Rinderer *et al.*, 2010) and grooming behaviour (Boecking and Spivak, 1999; Andino and Hunt, 2011).

Hygienic behaviour is a natural defence against brood diseases in which hygienic worker honey bees uncap cells containing brood that is dead or infected and remove the contents (Rothenbuhler, 1964; Rinderer *et al.*, 2010; Spivak, 1996). In this way, diseases such as chalk brood, American foulbrood and varroa infestation can be fully or partly controlled (Boecking and Spivak, 1999; Spivak and Gilliam, 1998). Despite a number of recent reviews on varroa resistance (Büchler *et al.*, 2010; Rinderer *et al.,* 2010; Carreck, 2011), there has been relatively little research on the role of hygienic behaviour in varroa control.

Colonies selected for hygienic behaviour using the Freeze Killed Brood (FKB) removal assay had fewer varroa mites than unselected commercial colonies (Ibrahim *et al.*, 2007; Ibrahim and Spivak, 2006; Harbo and Harris, 2001; Delaplane *et al.*, 2005). Colonies selected for Varroa Sensitive Hygiene (VSH), which have greater mite removal

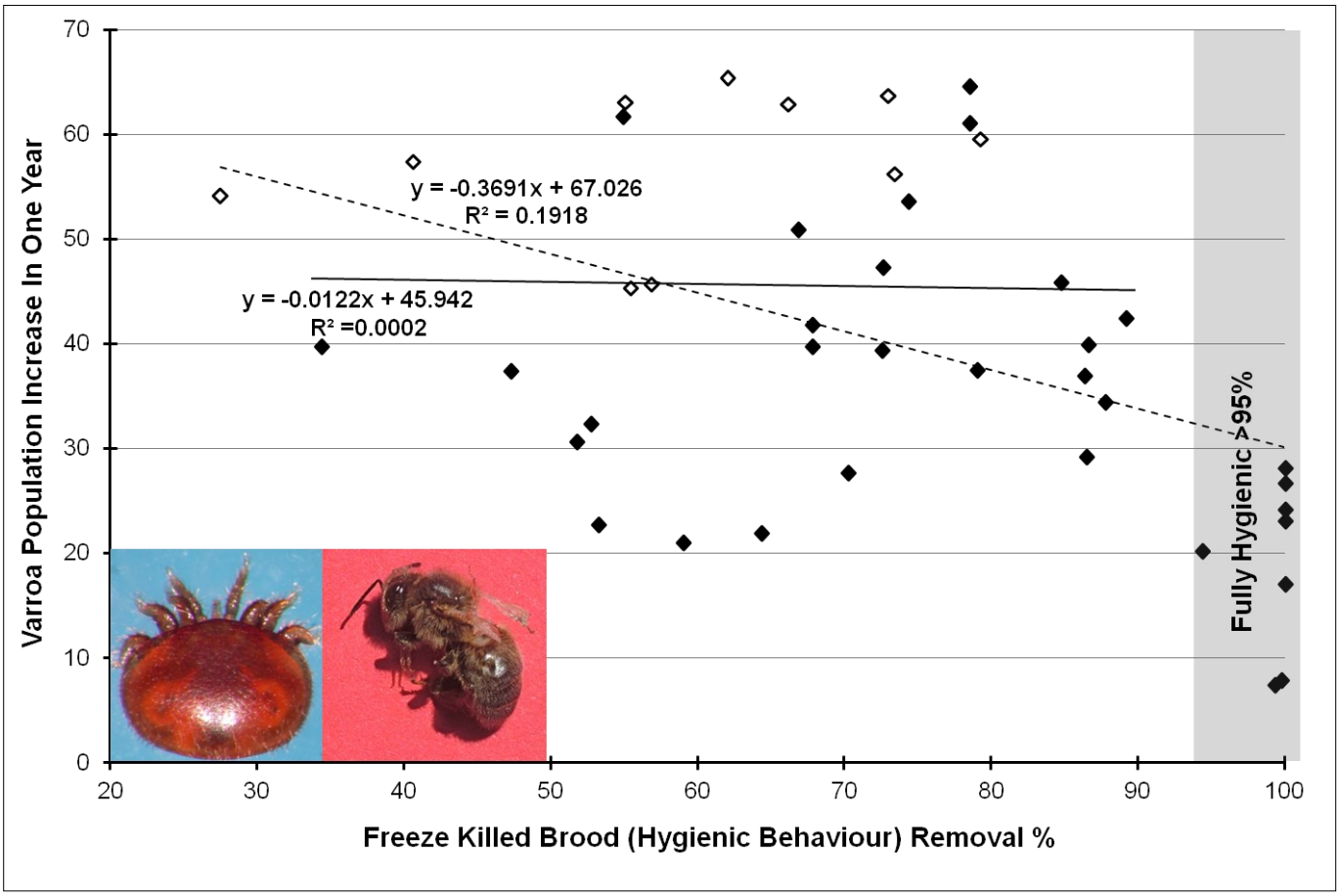

Fig. 1. Proportional increase of varroa population from 12 January to 12 December 2013 in the 42 study colonies as a function of Freeze Killed Brood removal. Colonies with workers showing symptoms of deformed wing virus are shown as open symbols. The photos show (left) an adult female varroa mite and (right) an adult worker bee with shrivelled wings, an overt symptom of DWV.

than hygienic colonies selected with the FKB bioassay (Ibrahim and Spivak, 2006; Delaplane *et al.*, 2005), show reduced varroa population growth (Peng *et al.,* 1987; Ibrahim and Spivak, 2006). Schöning *et al.* (2012) showed that hygienic colonies uncap cells containing a female varroa mite seven days after capping. However, only cells containing a mother mite infected with deformed wing virus (DWV) were uncapped and cleaned out. Cells with uninfected mites were not uncapped. In this study we quantified hygienic behaviour in honey bee colonies using the FKB assay to determine the effect of intercolony variation in FKB removal on varroa population increase and incidence of DWV over one year.

Material and methods

Study colonies and data collection

We studied 42 honey bee colonies in four apiaries within 20 km of the University of Sussex. The colonies were managed using normal beekeeping methods. Each was housed in a hive consisting of a single "Commercial" brood chamber (11 frames 43.8 cm x 25.4 cm; total volume 56.4 l), bottom board with mesh floor, inner cover and telescopic cover. Each hive was given a queen excluder and honey supers as needed, and the honey crop was removed in early August.

All colonies were treated with oxalic acid when broodless on 2 January 2013. In broodless colonies all the varroa are phoretic on adult bees so can be killed by oxalic acid (Gregorc and Planinc, 2001). Ten days later, which is sufficient time for the complete mortality effect of the oxalic acid (Al Toufailia *et al.*, 2015), a sample of *c.* 300 worker bees was taken from each colony, which were all still without sealed brood for varroa to enter, and frozen. The varroa mites were then washed from the sampled bees using a jet of water (Dietemann *et al.*, 2013; Al Toufailia *et al.*, 2015) to determine the initial number of mites per 100 bees. As the colonies were broodless when the sample was taken, this gave an estimate of the whole varroa population in each colony. No other treatments against varroa were used.

Starting on 19 August 2013, each colony was tested four times at weekly intervals using the Freeze Killed Brood removal assay (Spivak and Downey, 1998; Spivak and Reuter, 1998a,b; Bigio *et al.*, 2014) to

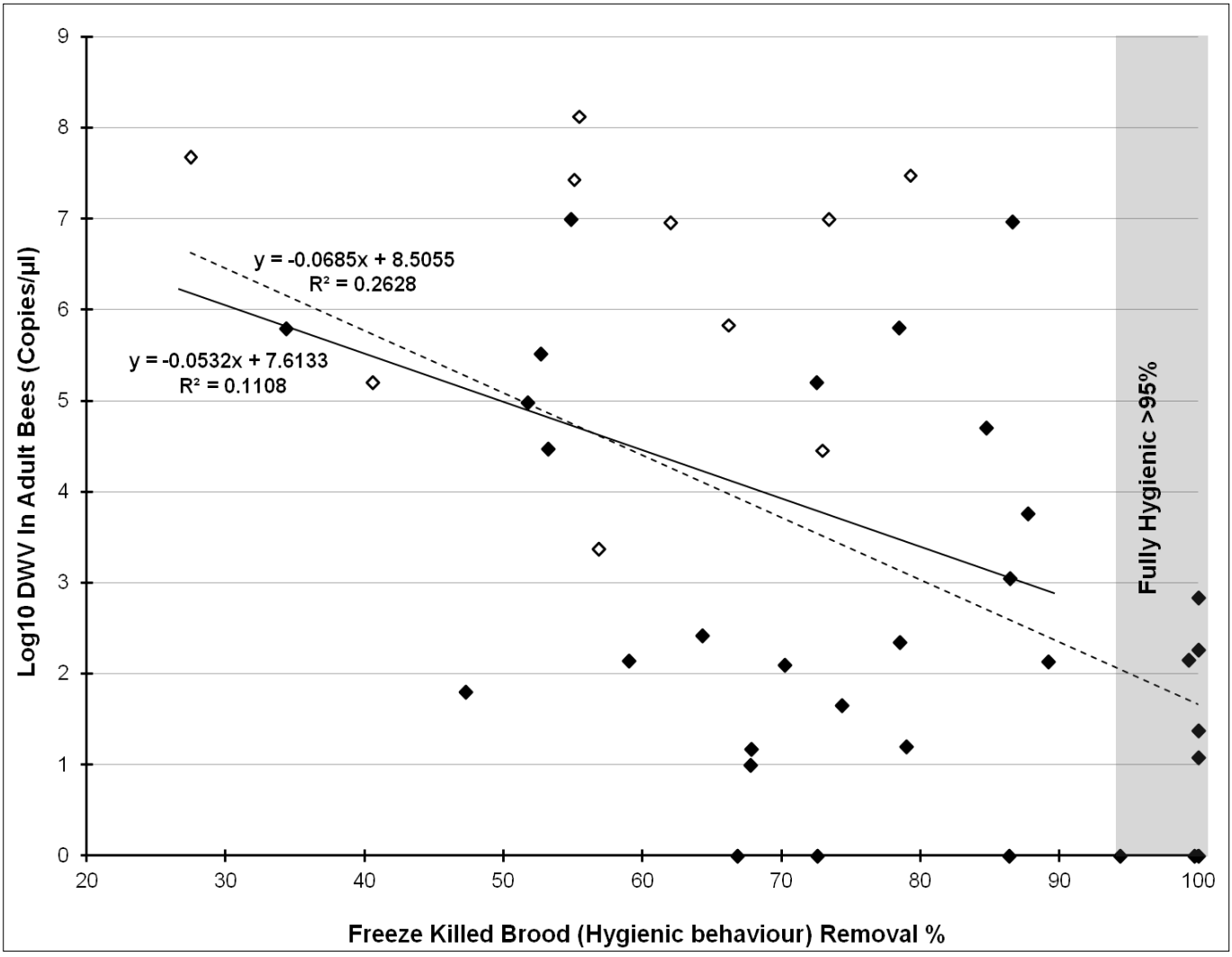

Fig. 2. Number of deformed wing virus RNA copies in adult bee samples collected on 12 December 2013, 11 months after treating with oxalic acid, in the 42 study colonies. Colonies that had some workers with overt symptoms of DWV (shrivelled wings) are shown as open symbols.

Table 1. DNA primer sequences used for quantitative PCR assays and for establishing standard curves.

Source	Primer name	Primer sequence	Product size	Reference
DWV	F-DWV R-DWV	5′-GGATGTTATCTCCTGCGTGGAA 5′-CTTCATTAACTGTGTCGTTGATAATTG	69bp	Gauthier *et al.*, 2007
Varroa.β.Actin	FV-β-Actin RV-β-Actin	5′- GTTCATCGGAATGGAGTCATGCGGT 5′- CCAGAGAGAACGGTGTTAGCGTACA	108bp	Francis *et al.*, 2013
Bee.β.Actin	F-β-Actin R-β-Actin	5′-TGCCAACACTGTCCTTTCTGGAGGT 5′- TTCATGGTGGATGGTGCTAGGGCAG	96bp	Francis *et al.*, 2013

quantify hygienic behaviour. In September 2013 each hive was inspected for the presence or absence of worker bees showing overt symptoms (shrivelled wings: see Fig. 1) of DWV. To do this, each frame of bees was viewed on both sides during a hive inspection. On 12 December 2013 a second sample of *c.* 300 worker bees was taken from each colony, frozen and used to estimate the final varroa population. The colonies were all broodless at this time.

Virus quantification

Analysis of DWV in worker bee and varroa samples followed previously used methods (Francis *et al.*, 2013). Briefly, *c.* 50 bees and

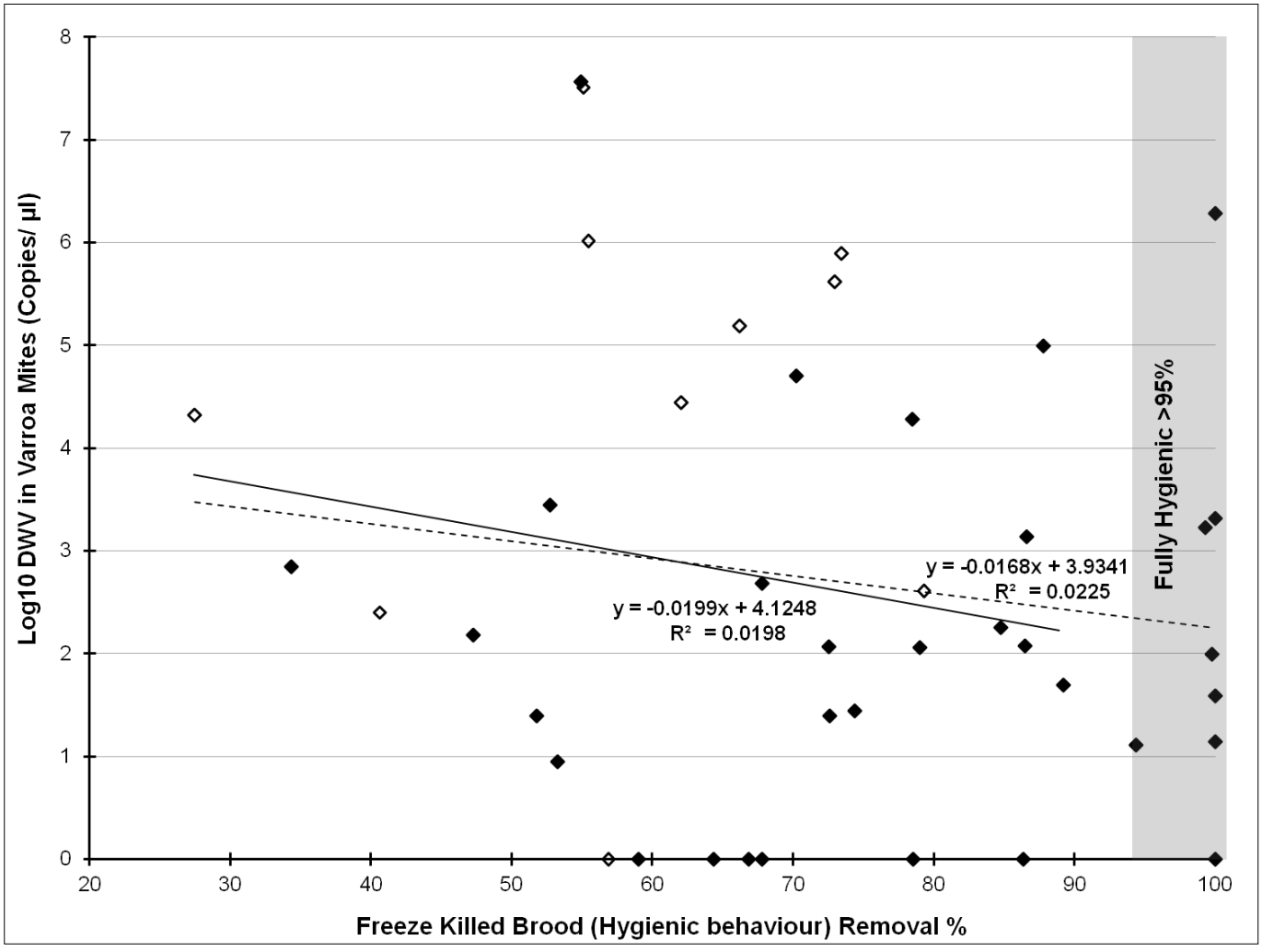

Fig. 3. Number of deformed wing virus RNA copies in varroa mite samples collected on 12 December 2013, 11 months after treating with oxalic acid, in the 42 study colonies. Colonies with workers showing overt symptoms of deformed wing virus are shown as open symbols.

10 mites from each of the December samples were placed into a 15 ml bottle together with 7 – 10 steel ball bearings. For the mites, 10 individuals per colony were placed in a 1.5 ml Eppendorf tube with 2 steel ball bearings and freeze-dried for three days at 0.009 hPa and -93 °C. After homogenisation of the samples, total RNA was extracted using NucleoMag 96 RNA kit (Machery- Nagel; Düren, Germany) on a Kingfisher Magnetic Extractor according to the manufacturer's guidelines.

A two-step real-time RT-PCR assay was used to quantify virus levels in the samples. Quantitative PCR amplifications were carried out on a vii7 apparatus (Applied Biosystems) in duplicate for each sample using SYBR® Green DNA binding dye (Applied Biosystems). Viral loads in each sample were quantified using methods for absolute quantification based on standard curves obtained through serial dilutions of known amounts of PCR amplicon (Francis *et al.,* 2013). Species specific β-Actin primers were included in the analysis, as an internal control for either honey bee or varroa samples. The primer sequences are shown in Table 1.

Statistical analysis

Data were analysed using the IBM SPSS statistical program version 20. If necessary, the response variable was log or arcsine transformed to meet the assumptions of ANOVA (Zuur *et al.*, 2010; Grafen *et al.*, 2002). Linear regression was then used to test for the effects of hygienic behaviour on varroa population increase and t tests and Fisher's exact tests for the effects of varroa population build up on the presence or absence of DWV symptoms. Descriptive statistics are given as mean ± standard deviation.

Results

Mean freeze-killed brood (FKB) removal, a measure of hygienic behaviour, ranged widely among the colonies from 27.5 % to 100 % with an overall mean of 72.6 %. Eight colonies had FKB removal of >95 %, a threshold commonly used to signify "fully hygienic" (Spivak and Downey, 1998).

Initial levels of varroa mites per 100 worker bees were low following oxalic acid treatment (mean & SD: 0.55 ± 0.34). Nearly one year later, this had increased 36.25 fold to 17.38 ± 7.25, on average (Fig. 1). Population increase in the number of varroa mites per colony was determined using this increase in the number of mites per 100 workers, combined with change in colony population, measured as the number of frames covered with bees at the time that worker samples were taken. For example, if the number of mites had increased from 0.5 to 20 per 100 workers, but the colony population had increased from four to five frames of bees, then the total varroa population increase was (20/0.5) x (5/4) = 50.

Overall, the mean increase in varroa population was 40.23 fold (range 7.4 – 65.4) (Fig. 1). The mean increase in the fully hygienic colonies was 19.37 (range 7.4- 28.2) versus 45.14 (range 21.1 – 65.4) in the non-hygienic colonies (<95 % FKB). Across all 42 study colonies, there was a significant negative relationship ($F=17.068$, $P<0.001$; $R^2= 0.19$) between FKB removal and varroa population increase (Fig. 1 dashed regression line). However, this effect was entirely due to the influence of the fully hygienic colonies. In the 34 colonies with <95 % FKB removal (range 27.5 – 90 %) there was no trend to lower varroa increase with higher FKB removal ($F=0.006$, $P=0.937$; $R^2= 0.0002$; Fig 1 solid regression line).

None of the 14 colonies with >80 % FKB removal had workers with shrivelled wings, an overt symptom of DWV, whereas 10 of the 28 (36 %) colonies with <80 % FKB removal did. This difference is significant (P = 0.017, Fisher's Exact Test, two tailed). The mean final number of varroa per 100 worker bees was greater in colonies with overt DWV symptoms (28.23±2.27) than without (24.80±5.56) ($F=8.68$; $P=0.005$). Colonies with DWV symptoms also had significantly greater varroa build up (57.36±7.19) than those without symptoms (34.87±14.36) ($F=4.36$; $P=0.043$).

Virus quantification

Overall, the range of viral RNA levels in the pooled worker bee samples in December 2013 was 0 - $1.3x10^8$ copies/µl (Fig. 2). The average level in the 8 fully hygienic colonies (>95 % FKB removal) was 8.6 x 10^2 copies/µl *vs.* $8.2x10^6$ copies/µl in the 34 non-hygienic colonies (<95%). Across all 42 study colonies, there was a significant negative relationship ($F=14.258$, $P<0.001$; $R^2= 0.26$) between FKB removal and viral RNA levels in worker bees (Fig. 2. dashed regression line). However, this effect was entirely due to the influence of the fully hygienic colonies. In the 34 colonies with <95% FKB removal (range 27.5 – 90 %) there was a trend to lower viral level increase with higher FKB removal but the relation was non-significant ($F= 3.989$, $P=0.06$; $R2= 0.1108$; Fig. 2. solid regression line).

The mean viral level in the varroa mites collected from the 8 fully hygienic colonies (> 95 % FKB removal) was $2.5x10^5$ copies/µl versus $21x10^5$ copies/µl in the 34 non-hygienic colonies (<95 %). Across all 42 study colonies, there was a non-significant relationship ($F=0.918$, $P= 0.34$; $R^2= 0.022$) between FKB removal and viral RNA levels in varroa mites (Fig. 3. dashed regression line). In the non-hygienic colonies with <95 % FKB removal, there was a non-significant relationship ($F= 0.647$; $P= 0.43$; $R2= 0.019$; Fig. 3. solid regression line).

Discussion

Our results show clearly that hygienic behaviour can be effective at reducing the one-year population growth of varroa in honey bee colonies. In particular, the fully hygienic colonies (>95 % FKB

removal) showed, on average, only 43 % of the varroa population growth of the non-fully hygienic colonies (<95 % FKB removal) (mean 19.37-fold, range 7.4 to 28.2, v 45.14-fold, range 20.1 to 65.4, respectively). The significant negative correlation between varroa build up and FKB removal was entirely due to the fully hygienic colonies (n = 8, >95 % FKB removal) having lower varroa build up than the colonies with FKB <95 % removal. There was no trend to lower varroa build up among the 34 colonies with FKB <95 % removal. The annual varroa increase we quantified is greater than the 12-fold estimate based on simulation modelling (Martin, 1998) but is within the wide range, 10-300 fold, found in previous empirical research (De Guzman *et al.*, 2007; Fries *et al.*, 1991; Kraus and Page, 1995). These earlier studies were carried out in different locations and conditions, and estimated varroa increase indirectly by counting mite fall onto the hive bottom board rather than directly, as in our study.

Our results also show that hygienic behaviour reduces the occurrence of DWV in that none of the 14 colonies with >80 % FKB removal showed overt symptoms of DWV. That is, the presence of workers with shrivelled wings. The fact that lower levels of FKB removal (80 v 95%) seemed to be effective in controlling DWV but were not effective in slowing varroa population growth may be because hygienic behaviour particularly targets capped cells containing a mother mite infected with DWV (Schöning *et al.,* 2012; Ibrahim and Spivak, 2006). It seems that DWV transmission to the immature bee results in odours that trigger cell uncapping.

Intercolony variation in the number of viral RNA copies show that the highly hygienic colonies (FKB removal > 95 %) had much lower levels of DWV in worker bees, some 10,000 times less than in the non -hygienic colonies (FKB removal < 95 %; $8.6x10^2$ versus $8.2x10^6$ copies/1 µl extract; Fig. 2.). However, varroa mites from hygienic colonies had only 8 times less viral RNA than those from non-hygienic colonies ($2.5x10^5$ versus $21x10^5$; Fig. 3.). It appears, therefore, that hygienic behaviour is more effective at reducing levels of DWV in worker bees than in the mites. Our study does not reveal why this is the case. Previous research has shown that hygienic bees remove worker pupae infected with DWV by varroa (Schöning *et al.*, 2012). This would tend to reduce virus levels more in worker bees than in mites, as infected pupae are killed while the female mite is probably not killed. However, this process could also reduce virus levels in mites, as infected mites would not be as successful in breeding as uninfected mites.

Overall, our results are encouraging to beekeepers, as they demonstrate that hygienic behaviour, which is a heritable and natural form of disease resistance, can reduce the build up of varroa mites and the incidence of DWV. There is now evidence that hygienic behaviour is beneficial against four honey bee pests and diseases (varroa, DWV, chalkbrood, American foulbrood (Boecking *et al.,* 2000; Boecking and Spivak, 1999; Spivak and Gilliam, 1998). Our results also support the advice given by Spivak and Reuter (2001) that hygienic colonies should require fewer additional treatments against varroa, possibly only annual treatment. Under the conditions of southern England, where our colonies were studied, it seems that hygiene combined with winter treatment of broodless hives with oxalic acid (Al Toufailia *et al.,* 2015) is sufficient for one year. Our results show that breeding hygienic bees is worthwhile for beekeepers, and support the recommendation of Spivak and Downey (1998) that an FKB removal of 95 % is a suitable criterion for "fully hygienic" colonies. Our results suggest, however, that it is possible that slightly lower levels of FKB removal (>80 %) may also provide protection against DWV, even if these intermediate levels (80-95% FKB removal) do not reduce varroa population build up compared to even lower levels of hygiene.

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